Patent
Attorney Docket No. 1029650-000178

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of	)	**MAIL STOP**
	)	
Isozaki, Masashi et al.	)	Group Art Unit: 1633
	)	
Application No.: 10/594,427	)	Examiner: Schultz, James
	)	
Filed: July 17, 2008	)	Confirmation No.: 2940
	)	
For: Liposome Preparation	)	
	)	CONSIDERED: /JDS/ (03/26/2012)
	)	
	)	

DECLARATION BY AN INVENTOR UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Masashi ISOZAKI hereby declare the following:

1. I am a citizen of Japan

2. I hold the position of research manager at TERUMO KABUSHIKI KAISHA and have worked in the company since the year 1988.

3. I graduated from Tokyo University of Science with a master degree in Pharmacy.

4. I am expert in the art of Organic chemistry.

5. My recent publications include:

   - KUNIKAZU MORIBE, AI KOBAYASHI, KOJI NAKAMURA, MASASHI ISOZAKI, KENJIROU HIGASHI, KEIJI YAMAMOTO. Effect of Cationic Lipids on PEG Mobility on the Surface of Liposomes. 11th Liposome Research Days Conference (LRD2008). 19-22 July, 2008. Yokohama, Japan.
   - KEISUKE YOSHINO, SHIGENORI NOZAWA, MASASHI ISOZAKI, SEIGO SAWADA, IKUO KATO, TAKESHI MATSUZAKI. Irinotecan Preparation. US7,846,473

- MASASHI ISOZAKI, TETSURO KAWANISHI. Process for production of O-alkylated rapamycin derivatives. US7,193,078
- TETSURO KAWANISHI, MASASHI ISOZAKI. Process for preparing an o-alkylated rapamycin derivative and o-alkylated rapamycin derivative. US7,812,155
- KAZUHIRO SHIMIZU, MASASHI ISOZAKI, KAZUNORI KOIWAI. Amidine derivatives and drug carriers comprising the same. US6,228,391

6. I am a co-inventor of the above-identified patent application, and I am submitting this declaration in support of that application.

7. I understand that the Examiner has rejected claims 1-6, 8-12, and 14-17 under 35 U.S.C. § 103(a) as allegedly unpatentable over Harigai et al., "Preferential Binding of Polyethylene Glycol-Coated Liposomes Containing a Novel Cationic Lipid, TRX-20, to Human Subendothelial Cells via Chondroitin Sulfate,"18(9) Pharmaceutical Research 1284-1290 (September 2001) ("Harigai") in view of U.S. Patent No. 5,616,341 to Mayer et al. ("Mayer"). Office Action mailed November 5, 2010, Pages 2-7.

8. I have read and understand the Harigai and Mayer documents.

9. I understand that the issue to be considered under 35 U.S.C. § 103(a) is whether the prior art, taken as a whole, would have rendered the present invention obvious to a person of ordinary skill in the art at the time that the invention was made. In my opinion, the prior art did not render the invention obvious for the following reasons.

10. At the time that the invention was made, vesicles, and in particular, liposomes modified with a hydrophilic macromolecule had been developed for use in clinical practice. The modified liposomes achieved high retentivity in blood. A typical hydrophilic macromolecule used in such vesicles was a derivative of polyethylene glycol (PEG) attached to a lipid such as a phospholipid or cholesterol. This is described, *e.g.*, in paragraph [0003] of the application.

11. The conventional way of making liposomes, then and now, is described by Mayer. *See, e.g.,* Mayer at col. 19, Example 3. Liposome forming lipids are dissolved in a volatile organic solvent and then dried into a thin film. The film is then hydrated with an aqueous buffer solution. The hydrated lipids are typically processed through a series of freeze-thaw steps and extruded though a membrane having pores of a defined size to produce a uniform population of liposomes of the desired size.

12. None of the examples in Mayer demonstrates the production of PEG modified liposomes. However, Mayer does provide an example of the conventional method of making liposomes containing more than one lipid component. *See, e.g.,* Mayer at col. 20, Example 6. In this example, egg phosphatidylcholine (EPC) and cholesterol were mixed in chloroform, dried into a thin film, hydrated in aqueous solution, and then processed by freeze-thaw and extrusion through a membrane.

13. An example of the conventional method of preparing liposomes modified with PEG can be found in U.S. Patent Number 5,213,804 (Martin). *See, e.g.,* Martin at cols. 32-33, Example 10. Here, again, vesicle-forming lipids including a PEG modified lipid were dissolved in chloroform, dried to a thin lipid film, hydrated with an aqueous solution, and processed through freeze-thaw cycles and extrusion through a membrane.

14. This conventional method of producing liposomes is simple and reliable. However, vesicles containing lipids modified with a hydrophilic macromolecule suffered from a lack of stability if the interior phase was maintained at an acidic pH. This becomes particularly problematic when the vesicles are to be used to deliver drugs that are unstable at neutral pH or higher, as described, *e.g.,* in paragraph [0007] of the application. Such drugs should be maintained in vesicles or liposomes that have an acidic interior environment.

15. My co-inventors and myself studied this problem and discovered that the instability of the vesicles was caused by hydrolysis of the lipids that comprise the vesicle membrane by the acidic interior environment. It was found that if the vesicles were prepared

in a manner such that the lipids modified with a hydrophilic polymer were only on the outside of the vesicle, then the storage capability of the vesicles is maintained even when the interior of the vesicles is acidic. This is described, *e.g.*, in paragraph [0008] of the application.

16. The conventional method of producing vesicles or liposomes that is described by Mayer, and throughout the art, results in a more-or-less uniform distribution of lipid components on the interior and exterior faces of the lipid bilayer. This is because the lipid components of the liposome are uniformly mixed together by being dissolved in an organic solvent at the start of the process.

17. In contrast to the conventional method, Harigai describes a method of introducing a hydrophilic polymer modified lipid to a liposome such that the hydrophilic polymer will be only on the outside surface. *See* Harigai at 1285, right column, "Preparation of Cationic Liposomes." In this method, liposomes are first prepared without a PEG lipid using the conventional method (substituting sonication for freeze-thaw). Then, in an added step, PEG-modified DSPE lipid (PEG-DSPE) was incubated at various concentrations with the prepared liposomes.

18. The method described by Harigai requires an additional separate step of incubating with PEG-DSPE after the liposomes are prepared according to the conventional procedure in order to introduce a PEG-modified lipid. For this reason, prior to the discovery that led to our invention, it would not have been obvious to deviate from the conventional method of Mayer—or Martin—by using the method of Harigai. Adding more steps to a process inherently increase the cost of a process in terms of time, effort, materials, risk of error, and efficiency. It would not be obvious to increase the number of steps in a process without an expectation that the product would have advantages that were of greater value than the costs imposed by the added steps.

19. There are several ways of introducing a modification only to the outside surface of a liposome. However, introducing a modification only to the outside surface of a

liposome always requires performing additional steps beyond the conventional method of liposome preparation, regardless of how the modification is introduced. Consequently, for any specific case, it would not be obvious to make a liposome having a modification that is only on the outside surface unless there is some specific reason to make it that way. No such reason can be found in Mayer and/or Harigai, or generally in the knowledge in art as a whole at the time that the invention was made.

20. My co-inventors and I discovered that hydrolysis by an acidic interior environment of the lipids that comprise a vesicle membrane which is modified by a hydrophobic polymer could be substantially prevented if the modification is only to the outside. The following experiments were conducted or supervised by myself and/or my co-inventors and demonstrate the effect of the invention.

Materials

21. The following materials were used.

- Hydrogenated soybean lecithin (HSPC; SPC3) manufactured by Lipoid having a molecular weight of 790.
- Polyethylene glycol 5000-phosphatidyl ethanolamine (PEG_{5000}-DSPE) manufactured by NOF Corporation having a molecular weight of 6075.

Preparation Of Liposomes Containing Doxorubicin

22. ***Example 1*** is an example of the claimed liposome preparations. A lipid derivative of the hydrophilic macromolecule PEG_{5000}-DSPE (which is a distearoylphosphatidylethanolamine derivative of polyethylene glycol having a molecular weight of 5000 Dalton) was added to a liposome produced as described below under a low pH condition (pH 4) to provide a hydrophilic macromolecule (PEG chain) on the exterior surface of the exterior membrane of the liposome. Doxorubicin was added to the liposome by ion gradient method.

23. LUV liposomes were produced by the following method. Hydrogenated soybean phosphatidyl choline (HSPC) and cholesterol (Chol) were dissolved in t-butanol at a molar ratio (HSPC:Chol) of 54:46, and freeze dried to produce a mixture of the lipid membrane components. A 300 mM solution of citric acid and 300 mM solution of trisodium citrate was mixed. A solution for the interior aqueous phase was prepared by adjusting the pH to 4.0, and a solution for the exterior aqueous phase was prepared by adjusting the pH to 7.5.

24. 0.37 g of the mixed lipid produced as described above was weighed, and to this was added 10 mL of the solution of the interior aqueous phase. The mixture was placed in an incubator at 68° C. for 15 minutes for swelling, and agitated with Vortex to produced a crude dispersion of the liposome. Using an extruder (manufactured by Lipex Biomembranes) that has been heated to 68° C., the crude liposome dispersion was passed 5 times through a filter having a pore diameter of 200 nm; and after changing the filter to a filter having a pore diameter of 100 nm, the filtration procedure was repeated twice by using this filter (a filter with a pore diameter of 200 nmx5; a filter with a pore diameter of 100 nmx5; and a filter with a pore diameter of 100 nmx5) to thereby produce a LUV liposome dispersion. The sample after the extrusion was cooled in an ice bath.

25. The thus prepared liposome dispersion was subjected to gel filtration through a gel column (Sepharose 4 Fast Flow) that had been fully substituted with the exterior aqueous phase to thereby obtain a pH gradient. The sample after the gel filtration was cooled in an ice bath.

26. The lipid in the liposome after the gel filtration was quantitatively evaluated (quantitative determination of the HSPC). Based on the HSPC concentration calculated in the quantitative determination of the HSPC, PEG_{5000}-DSPE (manufactured by NOF Corporation) was added to a concentration of 1.0% by mole, and the mixture was agitated at 60° C. for 30 minutes to thereby introduce the PEG_{5000}-DSPE.

27. The amount of the doxorubicin hydrochloride was calculated based on the HSPC concentration that was calculated in the quantitative determination of the HSPC so that the ratio of the doxorubicin hydrochloride to the HSPC was 0.2 (w/w), and the required amount of the doxorubicin hydrochloride was weighed according to the calculation. A doxorubicin hydrochloride solution at 10 mg/mL was prepared using 10% sucrose solution (pH 9.0). A predetermined amount of the doxorubicin hydrochloride solution (1 0 mg/mL) was added to the liposome dispersion, and the mixture was stirred at 60° C. for 60 minutes to thereby introduce the doxorubicin hydrochloride in the liposome. The sample after the doxorubicin hydrochloride introduction was cooled in an ice bath. Gel filtration was conducted using a column (Sepharose 4 Fast Flow having a diameter of 2.8 cm and a length of 20 cm) fully substituted with 10% sucrose (pH 6.5) to remove the doxorubicin hydrochloride that had not been trapped in the liposome.

28. The phospholipid of the liposome was quantitatively determined using Phospholipid C-Test Wako manufactured by Wako Pure Chemical. Concentration of the doxorubicin encapsulated in the liposome was determined by measuring absorbance at 480 mn using a spectrophotometer for the solution prepared by adding 2 mL of methanol to 40 f.LL of the doxorubicin liposome. The liposome size was measured by diluting 20 µL of the liposome dispersion with physiological saline to 3 mL, and measuring average liposome diameter using Zetasizer 3000HS (Malvern Instruments). The liposome produced is shown in Table 1.

29. ***Comparative Example 1*** is a preparation of liposomes outside the scope of the present invention. The liposome preparation was produced by using the liposome components which are the same as those of Example 1 using a conventional method. The hydrophilic macromolecule (PEG chain) was distributed on both sides of the inner and outer layers of the liposome bilayer by introducing the lipid derivative of the hydrophilic macromolecule (PEG_{5000}-DSPE) simultaneously with the liposome formation.

30. More specifically, 0.37 g of the lipid mixture which was the same as the one used in Example 1 (HSPC:Chol=54: 46) was weighed, and based on the HSPC concentration of the lipid mixture, 0.073 g ofPEG_{5000}-DSPE was weighed so that the PEG5000-DSPE was at 2.0% by mole. After adding 1 mL of ethanol 1 ml, the mixture was dissolved in an incubator at 65° C. for 30 minutes. After confirming the complete dissolution, 10 mL of the interior aqueous phase was added, and the mixture was agitated by heating to 65° C. for 60 minutes to prepare the crude liposome dispersion. This crude liposome dispersion was processed with the extruder by repeating the procedure of Example 1, and the sample after the extrusion was cooled in an ice bath.

31. The thus prepared liposome was subjected to gel filtration by repeating the procedure of Example 1 through a gel colunm (Sepharose 4 Fast Flow) that had been fully substituted with the exterior aqueous phase to thereby obtain a pH gradient. The sample after the gel filtration was cooled in an ice bath.

32. The lipid in the liposome after the gel filtration was quantitatively evaluated (quantitative determination of the HSPC). Based on the thus determined HSPC concentration, amount of the doxorubicin hydrochloride was calculated so that the ratio of the doxorubicin hydrochloride to the HSPC was 0.2 (w /w). Based on the thus calculated amount, required amount of the doxorubicin hydrochloride was weighed, and doxorubicin hydrochloride solution at 10 mg/mL was prepared using 10% sucrose (pH 9.0).

33. By repeating the procedure of Example 1, a predetermined amount of the doxorubicin hydrochloride solution (10 mg/mL) was added to the liposome dispersion for introduction of the doxorubicin hydrochloride, and the doxorubicin hydrochloride that had not been encapsulated in the liposome was removed.

34. The amount of the phospholipid in the liposome, amount of the doxorubicin hydrochloride encapsulated in the liposome, and the liposome size were measured by repeating the procedure of Example 1. The liposome produced is shown in Table 1.

TABLE 1

	Membrane constitution (mole ratio)	Liposome size, nm	Amount of drug loaded, mole drug/ mole lipid
Example 1	HSPC:Chol:PEG_{5000}-DSPE = 54:46:1	118.8	0.11
Comparative Example 1	HSPC:Chol:PEG_{5000}-DSPE = 54:46:2	112.9	0.13
Example 2	SM: Chol:PEG_{5000}-DSPE = 54:46:0.75	107.4	0.12

Experimental Methods

35. ***Demonstration of Hydrolysis of PEG_{5000}-DSPE.*** Test Example 1 shows the difference in stability of the lipid derivative of a hydrophilic macromolecule (PEG_{5000}- DSPE) under acidic conditions < ph 5 versus neutral conditions > ph5.

36. PEG_{5000}-DSPE (manufactured by NOF Corporation) was dissolved in each of the following 4 buffers to a concentration of 5 mg/mL, and the solution was heated at 65° C. for 90 minutes. PEG_{5000}-DSPE was also dissolved in the same buffer to a concentration of 10 mg/mL, and the solution was stored at 40° C. for 1 week.

- Buffer (1): ammonium sulfate (250 mM)
- Buffer (2): L-Histidine (10 mM), 10% sucrose, pH 6.5
- Buffer (3): citric acid (300 mM), pH 4.0
- Buffer (4): citric acid (300 mM), pH 7.5

37. 10 µL of the thus stored solution was spotted at a position 1 cm above the lower end of the silica gel thin layer of 20 cm x 20 cm. This silica gel thin layer was then placed in a glass container that had been preliminarily equilibrated with the developing solvent which was a mixed solution of chloroform, methanol, and ammonia (28):(85:14:1) for

development with the developing solvent for about 15 cm, and the degradation product was searched by iodine color development.

38. The results of this thin layer chromatography (TLC) are shown in FIG.1 to 2. FIG.1 shows the results of the TLC for the PEG_{5000}-DSPE solution which had been heated to 65° C. for 90 minutes. FIG. 2 shows the results of the TLC for the PEG_{5000}-DSPE solution which had been heated to 40° C. for 1 week.

FIG.1

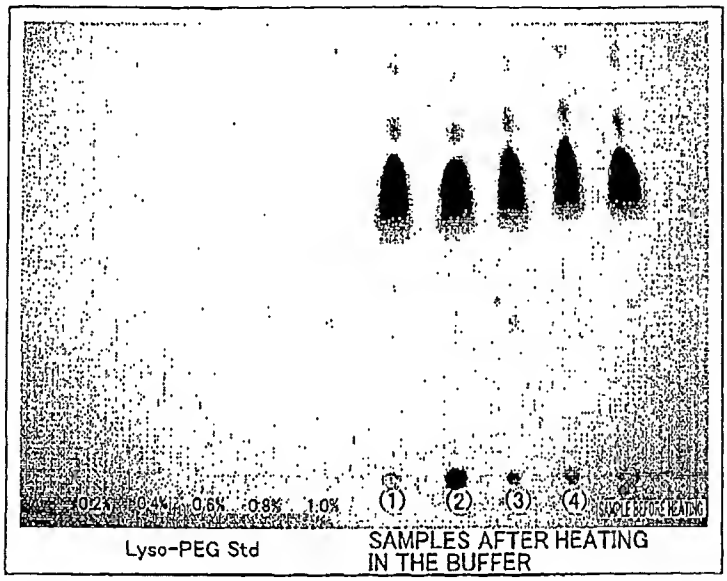
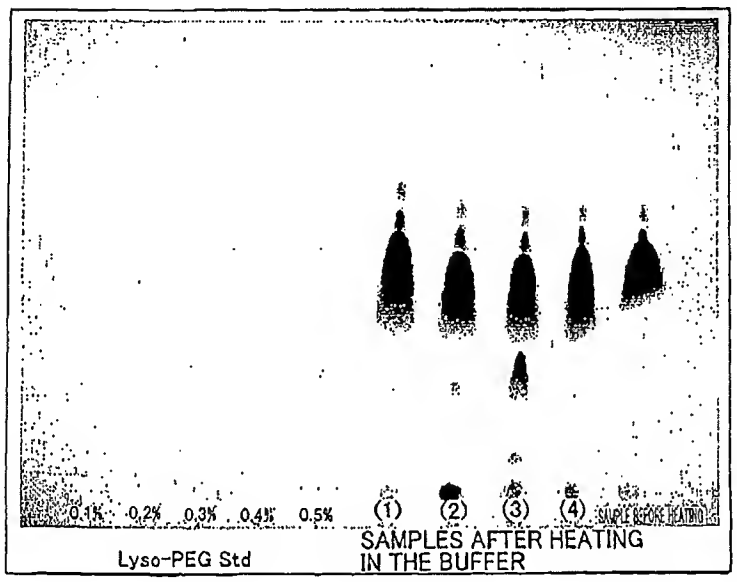


FIG. 2



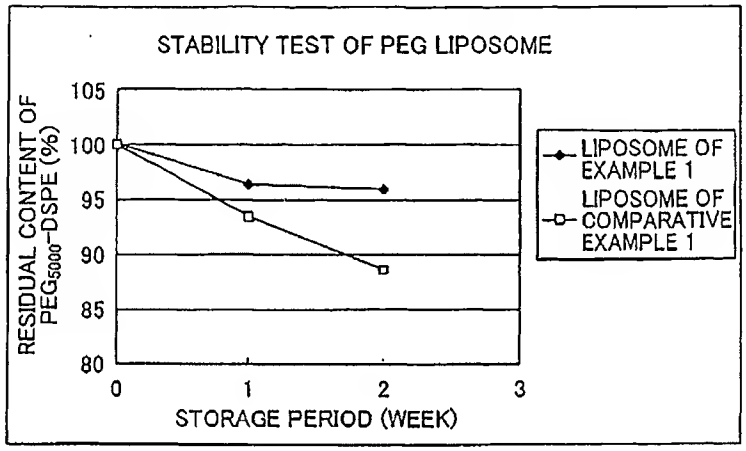
39. No increase in the spot was observed at the position of the degradation product (lyso-lipid) before and after the heating for the PEG_{5000}- DSPE that had been dissolved in the buffer at a pH of 5 or higher. An increase in a spot representing degradation of the PEG_{5000}- DSPE to lyso-PEG was evident in the case of the PEG_{5000}-DSPE that had been dissolved in the buffer at pH 4.

40. The data show that the PEG_{5000}-DSPE is degraded when heated under acidic conditions (buffer (3), citric acid, pH 4.0). This in turn means that, when the liposome is produced by a method in which the PEG_{5000}-DSPE is kept under acidic buffer conditions as in the case of Comparative Example 1, the PEG_{5000}-DSPE would be expected to degrade during manufacture or storage.

41. ***Comparison of Degradation Behavior Between the Liposomes of Example 1 and Comparative Example 1*** From the data of Test Example 1, one would expect that because in the liposome produced by the method of Comparative Example 1, the internal aqueous phase is acidic, the PEG_{5000}-DSPE that is in contact with the internal aqueous phase is also expected to degrade.

42. For Test Example 2, liposomes produced in the Example 1 and the Comparative Example 1 were stored at 40° C. for 1 week or 2 weeks, and the liposomes were then evaluated for their residual content of the PEG_{5000}-DSPE by HPLC. The results are shown in FIG. 3 by the residual content of the PEG_{5000}-DSPE for the PEG liposomes stored at 4° C.

FIG. 3



43. The results of the Test Example 2 demonstrate that the residual content of the PEG_{5000}-DSPE decreased in the case of the liposome of the Comparative Example 1 indicating the degradation of the PEG_{5000}-DSPE whereas no significant change in the residual content was observed in the case of the liposome of the present invention produced in Example 1 indicating reduced degradation of the PEG_{5000}- DSPE.

Conclusions

44. The data show that the degradation of the lipid components of a liposome having an interior phase at pH < 5.0 can be substantially reduced by providing a liposome in which a hydrophilic polymer is located only on the exterior of the liposome. When the degradation of the PEG_{5000}-DSPE is prevented, destabilization of the lipid bilayer, leakage of the drug that is contained in the liposome, aggregation of the liposome, decrease in the effect of preventing adsorption of the liposome to plasma protein or opsonin protein, loss of

liposome stability in blood, and other problems associated with the degradation of the PEG_{5000}-DSPE are also prevented.

45. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-captioned patent application or any patent issuing thereon.

Dated: 2012. 2. 20

Signed: Masashi Isozaki

Masashi ISOZAKI